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ORIGINAL RESEARCH

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Phenotypic and genotypic identification of class C and D β-lactamases in clinical isolates of *Pseudomonas aeruginosa*: a cross-sectional study

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Abstract

Background and Aims: The emergence of *Pseudomonas aeruginosa* (*P. aeruginosa*) antibiotic resistance is an important public health problem worldwide that can negatively affect infection control. Therefore, obtaining knowledge about antibiotic resistance mechanisms is necessary for infection control policies. This study aimed to determine the frequency of class C and D β-lactamases in *P. aeruginosa* strains isolated from patients referred to Ardabil hospitals using phenotypic and genotypic tests.

Methods: Phenotypic detection of β-lactamases including AmpC cephalosporinase, oxacillinase (OXA)-type extended-spectrum β-lactamases (ESBLs), and OXA-type carbapenemases were performed using the disk diffusion-based methods. Amplification of genes encoding classes C (*ampC* and *FOX* genes) and D (*OXA-1*, *OXA-2*, *OXA-10*, *OXA-23*, and *OXA-48* genes) β-lactamases was performed using the polymerase chain reaction (PCR) method. A quantitative reverse transcription PCR (qRT-PCR) method was used to determine the expression level of the *ampC* gene among multiple drug-resistant and imipenem-resistant *P. aeruginosa* strains.

Results: In phenotypic tests, the prevalence of AmpC cephalosporinase, OXA-type ESBLs, and OXA-type carbapenemases were 52.5%, 7.2%, and 95.8%, respectively. In genotypic tests, the prevalence of *ampC*, *FOX*, *OXA-1*, *OXA-2*, *OXA-10*, *OXA-23*, and *OXA-48* genes were 100%, 0%, 4.3%, 60.8%, 42%, 29.7%, and 2.9%, respectively. In addition, the *ampC* gene overexpression was seen in 16 (33.3%) drug-resistant *P. aeruginosa* clinical isolates.

Conclusion: Given the presence of class C and D β-lactamases in clinical isolates of *P. aeruginosa* in Ardabil hospitals, early detection of these strains can help prevent the spread of resistant strains in hospital environments and subsequent treatment failure.

KEYWORDS

AmpC beta-lactamase, extended-spectrum β-lactamase, OXA-type β-lactamase, *Pseudomonas aeruginosa*

1 | INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a ubiquitous and opportunistic microorganism that is found in hospital environments, especially in intensive care units, and is considered as one of the most important etiological agents for hospital-acquired infections.[1–3] Effective therapeutic options against hospital-acquired infections due to *P. aeruginosa* have been faced with challenges due to the intrinsic, acquired and adaptive resistance mechanisms of the bacterium to various antibiotics.[4] *P. aeruginosa*'s intrinsic resistance to antibiotics occurs through outer membrane porins and efflux pumps, acquired resistance through horizontal transfer of resistance genes and mutations, as well as adaptive resistance through biofilm formation.[5] All above-mentioned mechanisms have been assessed in clinical isolates of *P. aeruginosa* obtained from hospital sources in Ardabil,[6–10] but there is no data on the prevalence of class C and D β-lactamase-producing *P. aeruginosa* strains. Enzymatic inactivation by β-lactamases is responsible for resistance to β-lactam antibiotics in clinically important Gram-negative bacteria.[11] According to the Ambler classification, class C β-lactamases, also known as AmpC β-lactamases, are cephalosporinases, which are encoded on the bacterial chromosome.[11] AmpC expression in *P. aeruginosa* and some *Enterobacteriaceae* organisms is induced when these bacteria are exposed to certain β-lactam antibiotics such as amoxicillin, ampicillin, imipenem, and clavulanic acid.[11] Furthermore, CMY, ACT, DHA, FOX, and MIR are plasmid-mediated enzymes, which belong to class C β-lactamases.[11] Class D β-lactamases in the Ambler classification, also known as oxacillinases or OXA-type β-lactamases, are either chromosomal or plasmid-encoded.[12] Substrate profiles for OXA-type β-lactamases are limited to cloxacillin, extended-spectrum cephalosporins, and carbapenems.[11] Obtaining knowledge about antibiotic resistance mechanisms is needed for infection control policies. Therefore, the present study explored the frequency of chromosomal or plasmid-encoded β-lactamases, i.e., AmpC and OXA-type β-lactamases, among clinical strains of *P. aeruginosa* isolated from patients referred to Ardabil hospitals.

2 | MATERIALS AND METHODS

2.1 | Collection of *P. aeruginosa* isolates

A total of 138 nonduplicate *P. aeruginosa* isolates were used in this cross-sectional study. Specimens were collected during June 2019 to January 2022 from outpatients or inpatients who referred to five Ardabil University of Medical Sciences affiliated hospitals. *P. aeruginosa* colonies were subcultured on cetrimide agar (Conda, Pronasida, Spain), and incubated at 37°C and ambient air for 24 h. Initial identification of the blue/green colonies was done using biochemical tests such as oxidase (+), catalase (+), urease (+), Gram staining (Gram-negative rod), OF (oxidative), IMViC (−, −, −, +), and TSIA (alkali/alkali (red/red)) and then confirmed with amplification of the 16S-23S rRNA internal transcribed spacer region.[13] Antimicrobial susceptibility testing of *P. aeruginosa* isolates for antibiotics suggested by CLSI (Clinical and Laboratory Standards Institute), i.e., piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, tobramycin, amikacin, netilmicin, ciprofloxacin, levofloxacin, norfloxacin, ofloxacin, and colistin, was done using the disk diffusion and agar dilution methods.[7,14] *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as the quality controls.

2.2 | Phenotypic detection of AmpC β-lactamase production

Phenotypic detection of AmpC β-lactamase production in *P. aeruginosa* was screened in all *P. aeruginosa* isolates using cefoxitin disk diffusion method.[15] Isolates showing reduced susceptibility to cefoxitin, i.e., zone of inhibition growth ≤ 18 mm, were selected for the phenotypic confirmatory test of AmpC enzyme production using inhibitor-based method.[16] For this purpose, a cefoxitin disk (FOX, 30 μg) (Padtan Teb, Iran) was placed adjacent to a cefoxitin disk containing phenylboronic acid (Sigma-Aldrich, Germany) (30/300 μg), at a distance of 30 mm, on plate of Mueller-Hinton Agar (Conda, Pronasida, Spain), which was already inoculated with *P. aeruginosa* suspension (0.5 McFarland turbidity). After an overnight incubation, a zone of growth inhibition ≥5 mm around the cefoxitin-phenylboronic acid disk, compared to the cefoxitin disk alone, indicated AmpC β-lactamase production.

2.3 | Phenotypic detection of OXA-type ESBLs production

A combined double-disk synergy test was performed for evaluating the possible presence phenotypic of OXA-type extended-spectrum β-lactamases (OXA-type ESBLs) in *P. aeruginosa* isolates.[17] Briefly, ceftazidime (CAZ, 30 μg) plus ceftazidime-clavulanate (30/10 μg) (Padtan Teb, Iran) and cefotaxime (30 μg) plus cefotaxime-clavulanate (30/10 μg) (Padtan Teb, Iran) disks were placed 20 mm apart from center to center on Mueller-Hinton Agar medium. After an overnight incubation, a zone of growth inhibition ≥5 mm around the combination disks, compared to ceftazidime and cefotaxime disks alone, indicated the ESBLs production. However, as the presence of inducible chromosomal AmpC β-lactamase can obscure the ESBLs identification in *P. aeruginosa*, hence, a combined double-disk synergy test was performed again for AmpC positive and ESBLs negative isolates using an inhibitor of AmpC β-lactamase overproduction, i.e., phenylboronic acid. For this purpose, ceftazidime disk vs ceftazidime disk containing phenylboronic acid and ceftazidime-clavulanate disk vs ceftazidime-clavulanate disk containing phenylboronic acid were used and then a zone of inhibition growth ≥ 5 mm around the combination disks considered as the AmpC and ESBL positive *P. aeruginosa*.

2.4 | Phenotypic detection of OXA-type carbapenemase production

Phenotypic presence of OXA-type carbapenemase enzymes among imipenem-resistant isolates ($n = 78$) was performed by double-disk synergy test.[18] For this purpose, imipenem (10 µg) (Padtan Teb, Iran) plus imipenem-EDTA (10 µg/750 µg) (Cypress Diagnostics, Belgium) disks were placed 20 mm apart from each other on Mueller-Hinton Agar medium. After an overnight incubation, a zone of growth inhibition ≥7 mm around the imipenem-EDTA disk indicated carbapenemase production.

2.5 | Genotypic detection of AmpC and OXA-type β-lactamases producing *P. aeruginosa*

DNA extraction was carried out using the boiling method. Amplification of genes encoding class C (*ampC* and *FOX* genes) as well as D β-lactamases including OXA-type ESBLs (*OXA-2* and *OXA-10* genes), OXA-type carbapenemases (*OXA-23* and *OXA-48* genes), and broad-spectrum β-lactamase (*OXA-1* gene) were performed by the Polymerase Chain Reaction (PCR) method (Applied Biosystems™ Veriti™ Thermal Cycler). PCR reactions were done in a total volume of 25 µL (20 µL of PCR Master Mix (Ampliqon, Denmark), 2 µL of each primer (10 µmol/L) (Metabion, Germany), and 3 µL of extracted DNA). Amplification conditions for each gene were listed in Table 1. PCR products were visualized on 1% agarose gel using the electrophoresis technique and then the presence of desired genes was confirmed using the sequencing method.

2.6 | Expression of *ampC* gene

A quantitative reverse transcription PCR (qRT-PCR) method was used to determine the level of transcription of the *ampC* gene in multiple drug-resistant and imipenem-resistant *P. aeruginosa* strains ($n = 48$) by LightCycler® System (Roche Diagnostics). Each reaction was performed in a final volume of 20 µL containing 10 µL of SYBR Green PCR Master Mix without ROX™ (Ampliqon, Denmark), 2 µL of each primer (10 µmol/L) (Metabion, Germany), 1 µL of cDNA, and 7 µL of DEPC-treated water. Primer sequence and qRT-PCR conditions were presented in Table 1. In this study, total RNA was extracted from overnight bacterial cultures using the RNA extraction kit (Favorgen, Taiwan) and their concentration and quality were determined by NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). For cDNA synthesis, 1 µg of extracted RNA was used for reverse transcription reaction with oligo (dT) and random hexamer primers (50 µM) according to the manufacturer's instructions (YTA, Iran). The 30S ribosomal protein S12 (*rpsL* housekeeping gene) was served as the internal control. The wild-type *P. aeruginosa* ATCC 27853 was used as the susceptible reference strain. Analysis of the relative expression of *ampC* gene was done using the $2^{-\Delta\Delta Ct}$ method. The *ampC* gene was considered overexpressed when the transcriptional mRNA level was ≥10-fold higher than that of the reference strain.[26] All qRT-PCR reactions were performed in duplicate.

2.7 | Statistical analysis

Associations between different OXA-type genes and *ampC* gene expression with the phenotypic antibiotic resistance were assessed using the Chi-square test and SPSS software (version 16). A p-value of <0.05 was considered statistically significant.

3 | RESULTS

The prevalence of resistance of 138 *P. aeruginosa* strains to different antibiotics was as follows: piperacillin 49/138 (35.5%), piperacillin-tazobactam 39/138 (28.3%), ceftazidime 52/138 (37.7%), cefepime 56/138 (40.6%), aztreonam 17/138 (12.3%), imipenem 78/138 (56.5%), meropenem 55/138 (39.9%), tobramycin 51/138 (37%), amikacin 49/138 (35.5%), netilmicin 84/138 (60.4%), ciprofloxacin 66/138 (47.8%), levofloxacin 68/138 (49.3%), norfloxacin 66/138 (47.8%), ofloxacin 87/138 (63%), and colistin 16/138 (11.6%). Demographic information of the 138 collected *P. aeruginosa* isolates is presented in Table 2. According to the results of screening tests for AmpC β-lactamase production, all isolates (100%) revealed a reduced susceptibility to cefoxitin (zone of inhibition growth ≤18 mm) (Figure 1A). These results are in consistence with the PCR results where all *P. aeruginosa* strains showed the *ampC* (279 bp) gene (Figure 2). However, 52.5% (63 of 120) of these isolates were AmpC β-lactamase positive in the phenotypic confirmatory test (Figure 1A). It is noteworthy that the frequency of plasmid-mediated AmpC β-lactamase, i.e., *FOX* gene, among *P. aeruginosa* isolates was 0%.

The *ampC* gene expression was assessed among 48 multiple drug-resistant and imipenem-resistant *P. aeruginosa* strains using the qRT-PCR. As shown in Figure 3, an increased gene expression was detectable in all 48 *P. aeruginosa* isolates in comparison with the reference strain *P. aeruginosa* ATCC 27853. However, the *ampC* gene overexpression among 16 (33.3%) drug-resistant *P. aeruginosa* clinical isolates was significant (≥ 10-fold). There was a significant association between the increase in *ampC* gene expression and resistance to tested β-lactam antibiotics i.e., piperacillin ($p = 0.013$), piperacillin-tazobactam ($p < 0.001$), ceftazidime ($p = 0.048$), cefepime ($p = 0.008$), imipenem ($p = 0.001$), and meropenem ($p = 0.004$).

ESBL-positive strains were not detected among the 138 *P. aeruginosa* isolates in the combined double-disk synergy test when an inhibitor of AmpC β-lactamase, phenylboronic acid, was not used (Figure 1A). However, 10 (7.2%) of the tested strains were ESBL-positive in the presence of phenylboronic acid (Figure 1B). In the current study, the prevalence of genes encoding OXA-type ESBLs among *P. aeruginosa* isolates was as follows: *OXA-2* 60.8% (84 of 138) and *OXA-10* 42% (58 of 138). There was a significant association between the presence of *OXA-2* gene and resistance to ceftazidime ($p = 0.031$) and meropenem ($p < 0.001$) as well as between the

TABLE 1 Primer sequence along with the PCR and qRT-PCR conditions used in the present study.

Gene	Oligonucleotide sequence (5′ to 3′)	Gene amplification conditions	Product size (bp)	Reference
PCR				
ampC	F: AGATTCCCCTGCCTGTGC R: GGCGGTGAAGGTCTTGCT	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 57 °C for 30 s Extension at 72 °C for 1 min (34 cycles)	279	[19]
FOX	F: AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	Initial denaturation at 94 °C for 3 min (1 cycle) Denaturation at 94 °C for 30 s Annealing at 64 °C for 30 s Extension at 72 °C for 1 min (25 cycles)	190	[20]
OXA-1	F: AGCCGTTAAAATTAAGCCC R: CTTGATTGAAGGATTGGGCG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 55 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	909	[21]
OXA-2	F: GCCAAAGGCACGATAGTTGT R: GCGTCCGAGTTGACTGCCGG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 67 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	701	[21]
OXA-10	F: TATCGCGTGTCTTTCGAGTA R: TTAGCCACCAATGATGCCC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 56 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	775	[22]
OXA-23	F: CCTCAGGTGTGCTGGTTATTC R: CCCAACCAGTCTTTCCAAAA	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 58 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	513	[23]
OXA-48	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 56 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	438	[24]
ITS	F: TCCAAACAATCGTCGAAAGC R: CCGAAAATTCGCGCTTGAAC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 55 °C for 1 min Extension at 72 °C for 1 min (34 cycles)	181	[13]
qRT-PCR				
ampC and *rpsL*	F: AGATTCCCCTGCCTGTGC R: GGCGGTGAAGGTCTTGCT and F: GCTGCAAAACTGCCCGCAACG R: ACCGCAGGTGTCCAGCGAACC	Pre-incubation at 95 °C for 600 s (1 cycle) Amplification: 95 °C for 20 s; 58 °C for 20 s (*ampC*), 64 °C for 20 s (*rpsL*) (40 cycles); 72 °C for 30 s Melting: 95 °C for 10 s; 65 °C for 60 s (1 cycle); 97 °C for 1 s	279 and 250	[19, 25]

TABLE 2 Demographic information of the 138 collected *P. aeruginosa* isolates.

Data	*n* (%)	Phenotypic resistance profiles *n* (%)	Multidrug-resistant *n* (%)	Genotypic resistance profiles *n* (%)	AmpC β-lactamase overproduction *n* (%)
Sex					
Male	71 (51.4%)	PIP 24 (49%), TZP 15 (38.5%), CAZ 23 (44.2%), FEP 27 (48.2%), ATM 7 (41.2%), IMP 38 (48.7%), MEM 25 (45.5%), TOB 24 (47.1%), AMK 22 (31%), NET 14 (43.8%), CIP 35 (53%), LVX 36 (52.9%), NOR 36 (54.4%), OFX 44 (50.6%), CST 11 (68.8%)	34 (50.7%)	*OXA-1* 3 (50%), *OXA-2* 44 (52.4%), *OXA-10* 26 (44.8%), *OXA-23* 24 (58.5%), *OXA-48* 2 (50%)	9 (56.3%)
Female	67 (48.6%)	PIP 25 (51%), TZP 24 (61.5%), CAZ 29 (55.8%), FEP 29 (51.8%), ATM 10 (58.8%), IMP 40 (51.3%), MEM 30 (54.5%), TOB 27 (52.9%), AMK 27 (44.9%), NET 18 (56.3%), CIP 31 (47%), LVX 32 (47.1%), NOR 30 (45.5%), OFX 43 (49.4%), CST 5 (31.3%)	33 (49.3%)	*OXA-1* 3 (50%), *OXA-2* 40 (47.6%), *OXA-10* 32 (55.2%), *OXA-23* 17 (41.5%), *OXA-48* 2 (50%)	7 (43.8%)
Age					
0–12	5 (3.6%)	PIP 3 (6.1%), TZP 2 (5.1%), CAZ 1 (1.9%), FEP 2 (3.6%), ATM 0 (0%), IMP 4 (5.1%), MEM 2 (3.6%), TOB 1 (2%), AMK 1 (2%), NET 1 (3.1%), CIP 1 (1.5%), LVX 1 (1.5%), NOR 1 (1.5%), OFX 3 (3.4%), CST 1 (6.3%)	3 (4.5%)	*OXA-1* 0 (0%), *OXA-2* 2 (2.4%), *OXA-10* 1 (1.7%), *OXA-23* 0 (0%), *OXA-48* 0 (0%)	2 (12.5%)
13–30	9 (6.5%)	PIP 2 (4.1%), TZP 2 (5.1%), CAZ 2 (3.8%), FEP 2 (3.6%), ATM 2 (11.8%), IMP 2 (2.6%), MEM 2 (3.6%), TOB 2 (3.9%), AMK 2 (4.1%), NET 1 (3.1%), CIP 2 (3%), LVX 3 (4.4%), NOR 2 (3%), OFX 3 (3.4%), CST 0 (0%)	3 (4.5%)	*OXA-1* 0 (0%), *OXA-2* 2 (3.4%), *OXA-10* 2 (3.5%), *OXA-23* 2 (4.9%), *OXA-48* 0 (0%)	1 (6.3%)
31–45	21 (15.2%)	PIP 6 (12.2%), TZP 4 (10.3%), CAZ 7 (13.5%), FEP 6 (10.7%), ATM 3 (17.6%), IMP 12 (15.4%), MEM 8 (14.5%), TOB 7 (13.7%), AMK 7 (14.3%), NET 5 (15.6%), CIP 9 (13.6%), LVX 9 (13.2%), NOR 10 (15.2%), OFX 13 (14.9%), CST 0 (0%)	10 (14.9%)	*OXA-1* 2 (33.3%), *OXA-2* 10 (11.9%), *OXA-10* 8 (13.8%), *OXA-23* 5 (12.2%), *OXA-48* 0 (0%)	1 (6.3%)
46–60	38 (27.5%)	PIP 14 (28.6%), TZP 14 (35.9%), CAZ 15 (28.8%), FEP 16 (28.6%), ATM 7 (41.2%), IMP 23 (29.5%), MEM 14 (25.5%), TOB 13 (25.5%), AMK 14 (28.6%), NET 10 (31.3%), CIP 20 (30.3%), LVX 20 (29.4%), NOR 20 (30.3%), OFX 28 (32.2%), CST 8 (50%)	21 (31.3%)	*OXA-1* 1 (16.7%), *OXA-2* 25 (29.8%), *OXA-10* 17 (29.3%), *OXA-23* 10 (24.4%), *OXA-48* 0 (0%)	6 (37.5%)
61–75	42 (30.4%)	PIP 14 (28.6%), TZP 10 (25.6%), CAZ 16 (30.8%), FEP 19 (33.9%), ATM 3 (17.6%), IMP 24 (30.8%), MEM 16 (29.1%), TOB 16 (31.4%), AMK 14 (28.6%), NET 7 (21.9%), CIP 21 (31.8%), LVX 22 (32.4%), NOR 20 (30.3%), OFX 24 (27.6%), CST 3 (18.8%)	19 (28.4%)	*OXA-1* 1 (16.7%), *OXA-2* 28 (33.3%), *OXA-10* 19 (32.8%), *OXA-23* 19 (46.3%), *OXA-48* 3 (75%)	4 (25%)
>75	24 (16.7%)	PIP 10 (20.4%), TZP 7 (17.9%), CAZ 11 (21.2%), FEP 11 (19.6%), ATM 2 (11.8%), IMP 13 (16.7%), MEM 13 (23.6%), TOB 12 (23.5%), AMK 11 (22.4%), NET 8 (25%), CIP 13 (19.7%), LVX 13 (19.1%), NOR 13 (19.7%), OFX 16 (18.4%), CST 4 (25%)	11 (16.4%)	*OXA-1* 2 (33.3%), *OXA-2* 15 (17.9%), *OXA-10* 11 (19%), *OXA-23* 5 (12.2%), *OXA-48* 1 (25%)	2 (12.5%)

(Continues)

TABLE 2 (Continued)

Data	*n* (%)	Phenotypic resistance profiles *n* (%)	Multidrug-resistant *n* (%)	Genotypic resistance profiles *n* (%)	AmpC β-lactamase overproduction *n* (%)
Specimen					
Sputum	34 (24.6%)	PIP 19 (38.8%), TZP 14 (35.9%), CAZ 19 (36.5%), FEP 19 (33.9%), ATM 4 (23.5%), IMP 25 (32.1%), MEM 22 (40%), TOB 21 (41.2%), AMK 19 (38.8), NET 12 (37.5%), CIP 21 (31.8%), LVX 22 (32.4%), NOR 20 (30.3%), OFX 38 (25.3%), CST 0 (0%)	19 (28.4%)	*OXA-1* 2 (33.3%), *OXA-2* 25 (29.8%), *OXA-10* 25 (43.1%), *OXA-23* 14 (34.1%), *OXA-48* 3 (75%)	4 (25%)
Urine	70 (50.7%)	PIP 20 (40.8%), TZP 14 (35.9%), CAZ 20 (38.5%), FEP 25 (44.6%), ATM 9 (52.9%), IMP 38 (48.7%), MEM 21 (38.2%), TOB 21 (41.2%), AMK 19 (38.8%), NET 14 (43.8%), CIP 33 (50%), LVX 34 (50%), NOR 33 (50%), OFX 44 (50.6%), CST 13 (81.3%)	33 (49.3%)	*OXA-1* 2 (33.3%), *OXA-2* 41 (48.8%), *OXA-10* 23 (39.7%), *OXA-23* 20 (48.8%), *OXA-48* 1 (25%)	11 (68.8%)
Wound	15 (10.9%)	PIP 8 (16.3%), TZP 8 (20.5%), CAZ 10 (19.2%), FEP 9 (16.1%), ATM 3 (17.6%), IMP 10 (12.8%), MEM 8 (14.5%), TOB 6 (11.8%), AMK 8 (16.3%), NET 4 (12.5%), CIP 8 (12.1%), LVX 8 (11.8%), NOR 9 (13.6%), OFX 11 (12.6%), CST 1 (6.3%)	12 (17.9%)	*OXA-1* 2 (33.3%), *OXA-2* 9 (15.3%), *OXA-10* 7 (12.1%), *OXA-23* 5 (12.2%), *OXA-48* 0 (0%)	1 (6.3%)
Blood	18 (13%)	PIP 2 (4.1%), TZP 3 (7.7%), CAZ 3 (5.8%), FEP 3 (5.4%), ATM 1 (5.9%), IMP 5 (6.4%), MEM 4 (4.3%), TOB 3 (5.9%), AMK 3 (6. 1%), NET 2 (6.3%), CIP 4 (6.1%), LVX 4 (5.9%), NOR 4 (6.1%), OFX 10 (11.5%), CST 2 (12.5%)	3 (4.5%)	*OXA-1* 0 (0%), *OXA-2* 9 (10.7%), *OXA-10* 3 (5.2%), *OXA-23* 1 (2.4%), *OXA-48* 0 (0%)	0 (0%)
CSF	1 (0.7%)	PIP 0 (0%), TZP 0 (0%), CAZ 0 (0%), FEP 0 (0%), ATM 0 (0%), IMP 0 (0%), MEM 0 (0%), TOB 0 (0%), AMK 0 (0%), NET 0 (0%), CIP 0 (0%), LVX 0 (0%), NOR 0 (0%), OFX 0 (0%), CST 0 (0%)	0 (0%)	*OXA-1* 0 (0%), *OXA-2* 0 (0%), *OXA-10* 0 (0%), *OXA-23* 1 (2.4%), *OXA-48* 0 (0%)	0 (0%)
Hospital					
Imam Khomeini	67 (48.6%)	PIP 23 (46.9%), TZP 19 (48.7%), CAZ 24 (46.2%), FEP 24 (42.9%), ATM 8 (47.1%), IMP 33 (42.3%), MEM 23 (41.8%), TOB 20 (39.2%), AMK 21 (42.9%), NET 8 (25%), CIP 26 (39.4%), LVX 28 (41.2%), NOR 26 (39.4%), OFX 38 (43.7%), CST 7 (43.8%)	29 (43.3%)	*OXA-1* 2 (33.3%), *OXA-2* 43 (51.2%), *OXA-10* 26 (44.8%), *OXA-23* 21 (51.2%), *OXA-48* 4 (100%)	8 (50%)
Alavi	44 (31.9%)	PIP 21 (42.9%), TZP 19 (48.7%), CAZ 24 (46.2%), FEP 24 (42.9%), ATM 8 (47.1%), IMP 30 (38.5%), MEM 25 (45.5%), TOB 24 (47.1%), AMK 23 (46.9%), NET 21 (65.6%), CIP 25 (37.9%), LVX 25 (36.8%), NOR 25 (37.9%), OFX 31 (35.6%), CST 2 (12.5%)	26 (38.8%)	*OXA-1* 4 (66.7%), *OXA-2* 24 (28.6%), *OXA-10* 25 (43.1%), *OXA-23* 13 (31.7%), *OXA-48* 0 (0%)	5 (31.3%)
Imam Reza	15 (10.9%)	PIP 2 (4.1%), TZP 0 (0%), CAZ 4 (7.7%), FEP 6 (10.7%), ATM 1 (5.9%), IMP 11 (14.1%), MEM 5 (9.1%), TOB 5 (9.8%), AMK 3 (6.1%), NET 1 (3.1%), CIP 13 (19.7%), LVX 13 (19.1%), NOR 13 (19.7%), OFX 13 (14.9%), CST 4 (25%)	9 (13.4%)	*OXA-1* 0 (0%), *OXA-2* 10 (11.9%), *OXA-10* 4 (6.9%), *OXA-23* 7 (17.1%), *OXA-48* 0 (0%)	1 (6.3%)
Bu-Ali	4 (2.9%)	PIP 2 (2.9%), TZP 1 (2.6%), CAZ 0 (0%), FEP 1 (1.8%), ATM 0 (0%), IMP 2 (2.6%), MEM 1 (1.8%), TOB 1 (2%), AMK 1 (2%), NET 1 (3.1%), CIP 1 (1.5%), LVX 1 (1.5%), NOR 1 (1.5%), OFX 2 (2.3%), CST 2 (12.5%)	2 (3%)	*OXA-1* 0 (0%), *OXA-2* 2 (2.4%), *OXA-10* 1 (1.7%), *OXA-23* 0 (0%), *OXA-48* 0 (0%)	1 (6.3%)

TABLE 2 (Continued)

Data	*n* (%)	Phenotypic resistance profiles *n* (%)	Multidrug-resistant *n* (%)	Genotypic resistance profiles *n* (%)	AmpC β-lactamase overproduction *n* (%)
Qaem	1 (0.7%)	PIP 0 (0%), TZP 0 (0%), CAZ 0 (0%), FEP 0 (0%), ATM 0 (0%), IMP 0 (0%), MEM 0 (0%), TOB 0 (0%), AMK 0 (0%), NET 0 (0%), CIP 1 (1.5%), LVX 0 (0%), NOR 0 (0%), OFX 1 (1.1%), CST 0 (0%)	0 (0%)	*OXA-1* 0(0%), *OXA-2* 0 (0%), *OXA-10* 0 (0%), *OXA-23* 0 (0%), *OXA-48* 0 (0%)	0 (0%)
Fatemi	2 (1.4%)	PIP 0 (0%), TZP 0 (0%), CAZ 0 (0%), FEP 0 (0%), ATM 0 (0%), IMP 0 (0%), MEM 0 (0%), TOB 0 (0%), AMK 0 (0%), NET 0 (0%), CIP 0 (0%), LVX 0 (0%), NOR 0 (0%), OFX 1 (1.1%), CST 0 (%)	0 (0%)	*OXA-1* 0 (0%), *OXA-2* 0 (0%), *OXA-10* 0 (0%), *OXA-23* 0 (0%), *OXA-48* 0 (0%)	0 (0%)
Sabalan	5 (3.6%)	PIP 1 (2%), TZP 0 (0%), CAZ 0 (0%), FEP 1 (1.8%), ATM 0 (0%), IMP 2 (2.6%), MEM 1 (1.8%), TOB 1 (2%), AMK 1 (2%), NET 1 (3.1%), CIP 0 (0%), LVX 1 (1.5%), NOR 1 (1.5%), OFX 1 (1.1%), CST 1 (6.3%)	1 (1.5%)	*OXA-1* 0 (0%), *OXA-2* 5 (6%), *OXA-10* 2 (3.4%), *OXA-23* 0 (0%), *OXA-48* 0 (0%)	1 (6.3%)

Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; IMP, imipenem; LVX, levofloxacin; MEM, meropenem; NET, netilmicin; NOR, norfloxacin; OFX, ofloxacin; PIP, piperacillin; TOB, tobramycin; TZP, piperacillin-tazobactam.

presence of *OXA-10* gene and resistance to piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, imipenem, and meropenem ($p < 0.001$).

Among the 138 clinical isolates of *P. aeruginosa*, the prevalence of imipenem-resistant *P. aeruginosa* was 56.5% ($n = 78$). Based on the double-disk synergy test, 95.8% (46 of 48 imipenem-resistant strains) of *P. aeruginosa* isolates were carbapenemase producers (Figure 1C). In addition, the prevalence of genes encoding OXA-type carbapenemases was as follows: *OXA-23* 29.7% (41 of 138) and *OXA-48* 2.9% (4 of 138). There was no significant association between the presence of *OXA-23* and *OXA-48* genes and resistance to antibiotics ($p > 0.05$).

It is noteworthy that the prevalence of *P. aeruginosa* strains positive for *OXA-1* gene was 4.3% (6 of 138).

Frequency of genes encoding various β-lactamases among multiple drug-resistant *P. aeruginosa* strains ($n = 67$) was as follows: *OXA-1* (7.5%), *OXA-2* (65.1%), *OXA-10* (65.1%), *OXA-23* (34.8%), and *OXA-48* (4.5%). In addition, the frequency of genes encoding various β-lactamases among imipenem-resistant *P. aeruginosa* strains ($n = 78$) was as follows: *OXA-1* (7.6%), *OXA-2* (67.9%), *OXA-10* (64.1%), *OXA-23* (33.3%), and *OXA-48* (3.8%).

The accession numbers in the GenBank database for detected genes are ON920993 and OP651437 to OP651440.

4 | DISCUSSION

According to our previous study and current findings, the prevalence of *P. aeruginosa* strains resistant to multiple antibiotics, including multidrug-resistant (MDR), extremely drug-resistant (XDR), and pandrug-resistant (PDR) strains, is increasing in Ardabil hospitals.[2] A systematic review and meta-analysis estimated the prevalence of MDR *P. aeruginosa* in Iran was 58% (48.5% in this study).[27] These findings are in accordance with assessments of the Centers for Disease Control and Prevention that announced the emergence of MDR *P. aeruginosa* as a serious antibiotic resistance threat in the world.[28] Therefore, gathering precise epidemiological data on *P. aeruginosa* resistance mechanisms, especially the production of β-lactamases including AmpC, ESBL, and carbapenemase enzymes, is required for infection control policies and proper patient management. On the other hand, the importance of genotypic identification of these enzymes is due to the fact that many of β-lactamase-producing strains indicate a susceptible phenotype in antimicrobial susceptibility testing.[29]

Drugs of choice for the treatment of MDR *P. aeruginosa* infection are carbapenems.[18] However, the emergence of carbapenem-resistant *P. aeruginosa* strains is a serious threat to human health in the world.[30] In this study, the prevalence of imipenem-resistant *P. aeruginosa* was high (56.5%) which is similar to the prevalence of imipenem-resistant *P. aeruginosa* among the Iranian population (54%).[31] In addition to the low permeability of the outer membrane and the presence of efflux pumps, carbapenemase and AmpC β-lactamase enzymes are involved in resistance to carbapenems.[32]

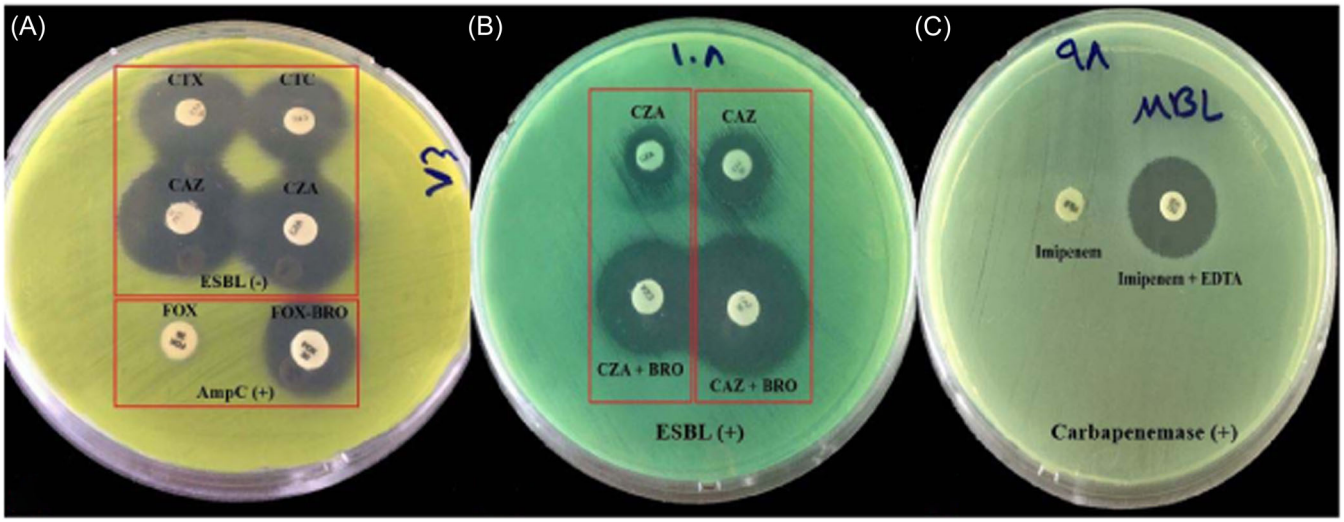

FIGURE 1 Phenotypic detection of AmpC β-lactamase (A), OXA-type ESBLs (B), and OXA-type carbapenemases (C) in clinical isolates of *P. aeruginosa*. FOX: cefoxitin, FOX-BRO: cefoxitin plus phenylboronic acid, CTX: cefotaxime, CTC: cefotaxime-clavulanate, CAZ: ceftazidime, CAZ-BRO: ceftazidime plus phenylboronic acid, CZA: ceftazidime-clavulanate, CZA-BRO: ceftazidime-clavulanate plus phenylboronic acid.

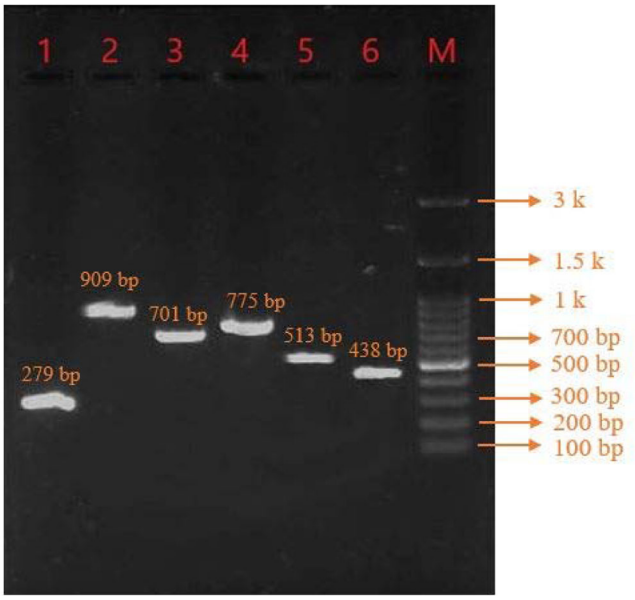

FIGURE 2 Results of gene amplification in the PCR method. Lane 1: *ampC* (279 bp), Lane 2: *OXA-1* (909 bp), Lane 3: *OXA-2* (701 bp), Lane 4: *OXA-10* (775 bp), Lane 5: *OXA-23* (513 bp), Lane 6: *OXA-48* (438 bp), and Lane M: ladder (100 bp).

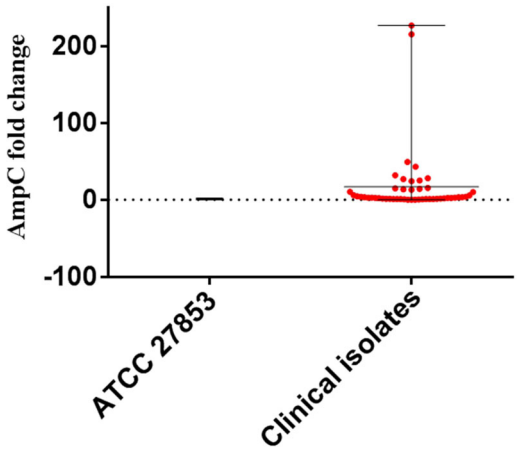

FIGURE 3 The overproduction of chromosomal AmpC cephalosporinase among drug-resistant *P. aeruginosa* clinical isolates. An increased level of the *ampC* gene transcription was observable among *P. aeruginosa* isolates. Each red circle represents a clinical strain.

However, the contribution of each of these resistance mechanisms is not clearly defined. In this study, the prevalence of AmpC-producing *P. aeruginosa* isolates was 52.5% in the phenotypic test, while other studies reported as follows: 46.9% by Noyal et al.,[32] 20.3% by Salimi et al.,[32] 36% by Chika et al.,[33] and 15.3% by Goel et al.[29] Differences in these results can be attributed to the difference in phenotypic detection techniques. There is no standard method recommended by the CLSI guideline to describe isolates producing AmpC β-lactamase. On the other hand, this study demonstrated that the overproduction of chromosomal AmpC cephalosporinase is involved in the emergence of *P. aeruginosa* isolates resistant to carbapenems and MDR strains. Similar results in accordance with our study were reported by others.[26] Lee et al. reported that AmpC overexpression (47.4%) was significantly associated with carbapenem resistance in *P. aeruginosa* isolates.[26] Cabot et al. estimated the prevalence of the *ampC* gene overexpression among nonsusceptible *P. aeruginosa* isolates to meropenem to be 60%.[34]

As mentioned above, the OXA-type carbapenemases, to a lesser extent, can be involved in the resistance of *P. aeruginosa* to carbapenems.[35] Among imipenem-resistant *P. aeruginosa* strains, 95.8% of the isolates were carbapenemase producers. The prevalence of carbapenemase in the phenotypic tests in our study was higher than that

reported in other studies from Iran,[32] India, USA, Spain, Korea, and Saudi Arabia.[35] The prevalence of genes encoding OXA-type carbapenemases in this study was 29.7% for *OXA-23* gene and 2.9% for *OXA-48* gene. Bahrami et al. reported that 12.5% of carbapenem-resistant *P. aeruginosa* strains were OXA-48-positive.[36]

Unlike AmpC β-lactamase and carbapenemases, ESBLs have no effect on carbapenems.[32] The most common ESBLs reported in *P. aeruginosa* are OXA-type β-lactamases. We found that 7.2% of *P. aeruginosa* isolates were ESBL-positive in the phenotypic test. The prevalence of ESBL-producing *P. aeruginosa* strains in other studies was as follows: Salimi et al. 12.5%,[32] Goel et al. 42.3%,[29] Tawfik et al. 69.4%,[35] and Aria et al. 61%.[37] OXA-type ESBLs are mainly derived from *OXA-2* and *OXA-10* genes.[35] In the current study, the prevalence of *OXA-2* and *OXA-10* genes were 60.8% and 42%, respectively. Tawfik et al. reported that the prevalence of OXA-10 ESBL in *P. aeruginosa* was 56% in Saudi Arabia.[35] In a study conducted by Jabalameli et al. in Iran, the most prevalent ESBL gene was *OXA-10* (70%).[38] Aria et al.[37] and Alipour et al.[39] reported that 32.3% and 87.6% of *P. aeruginosa* were positive for *OXA-10* gene, respectively.

The prevalence of *OXA-2* gene in other studies was as follows: Aria et al. 10.3%[37] and Alipour et al. 4.7%.[39]

5 | CONCLUSION

The current study reported the presence of *P. aeruginosa* strains producing AmpC cephalosporinase, OXA-type ESBLs, and OXA-type carbapenemases in clinical strains isolated in Ardabil hospitals. In addition, the *ampC* gene overexpression plays a key role in the emergence of carbapenem-resistant and MDR *P. aeruginosa* strains in our region. Given that *P. aeruginosa* is an important hospital-acquired pathogen, the implementation of genotypic methods as a routine test in hospital laboratories for the identification of β-lactamase-producing strains can help prevent the spread of resistance strains and ensuing treatment failure.

AUTHOR CONTRIBUTIONS

Nahid Mohammadnezhad: Investigation. **Maryam Nazari**: Investigation; Formal analysis; Methodology. **Seyyed Khalil Shokouhi Mostafavi**: Supervision. **Amirhossein Sahebkar**: Writing—review and editing. **Farzad Khademi**: Conceptualization; Methodology; Supervision; Writing—review and editing; Project administration; Formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

All data generated or analyzed during this study are included in this published article. Also, the data supporting the findings of this study are available in the NCBI GenBank repository, under the accession numbers: ON920993 and OP651437 to OP651440.

ETHICS STATEMENT

This research has been approved by the Research Ethics Committee of Islamic Azad Tehran Medical Sciences University with the code of IR.-IAU.PS.REC.1400.538. [https://ethics.research.ac.ir/ProposalCertificateEn.php?id=252752].

All methods were carried out according to relevant guidelines and regulations. Clinical isolates were collected from the hospital's bacterial repository solely for research purposes, and neither patient samples nor patient data were utilized in this study. Therefore, the requirement for informed consent from participants was waived by the Research Ethics Committee.

TRANSPARENCY STATEMENT

The lead author Farzad Khademi affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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